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14. ABSTRACT Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. The role of heparanase in breast cancer tumorigenesis remains unclear. In particular, whether HPR1 enzymatic activity is required for its stimulatory effect on tumor growth and initiation are not fully understood. Our earlier study showed that the C-terminus of HPR1, which lacks the enzymatic activity, was able to accelerate breast cancer formation in a somatic breast cancer mouse model since mice infected with RCAS-Neu virus plus RCAS-8C (a vector encoding the C terminus of HPR1), developed breast cancer significantly faster than that infected with RCAS-Neu plus RCAS-GFP (Green fluorescence protein) control virus. Here we report that the similar observations were made with PyMT oncogene-induced breast cancer, e.g. co-infection with RCAS-PyMT virus plus RCAS-8C virus induced breast cancer significantly faster than RCAS-PyMT virus plus RCAS-GFP. Enzymatically dead HPR1 had similar effect in stimulating breast cancer formation in the C-terminus of HPR1. In contrast, co-infection of TVA transgenic mice with RCAS-PyMT virus plus RCAS-HPR1 virus significantly delayed breast cancer formation than those infected with RCAS-PyMT virus plus RCAS-GFP control virus. Our results strongly suggest that HPR1 promotes tumor growth independent of its enzymatic activity, and that the enzymatic activity of HPR1 antagonizes the tumor initiating activity of HPR1.					
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Introduction

Heparanase-1 (HPR1) is an endoglycosidase overexpressed in many malignancies including breast cancer (1; 2). Previous studies suggest that the enzymatic activity of HPR1 can promote tumor angiogenesis and growth by degrading extra cellular matrix and releasing the growth factors. Using a xenograft mouse model, Flux et al. (3) reported that the full-length of the HPR1 gene stimulates tumor growth less effectively than the enzymatically inactive HPR1 gene, suggesting that HPR1 enzymatic activity may be disposable for its effect on stimulating tumor growth. Since the C-terminus of HPR1 can activate the PI-3 kinase pathway and induce endothelial and tumor cell migration independent of its enzymatic activity, it is not clear whether its enzymatic activity or C-terminus or both contribute to breast tumor initiation and growth. The goal of this project is to dissect the effect of HPR1 enzymatic activity and HPR1 C-terminus epitope on breast tumor initiation in a clinically relevant mouse breast cancer model. We proposed to determine if HPR1 knockdown will suppress or accelerate breast tumor initiation mediated by three oncogenes, PyMT, Neu and Wnt, and whether HPR1 C-terminus or an enzymatically dead HPR1 can stimulates breast tumor initiation, whereas full-length HPR1 has no effect or is less effective in stimulating breast tumor initiation and progression.

Experimental procedures and results

The effect of HPR1 on the PI-3 kinase and MAP kinase pathway activation.

Three RCAS vectors containing a full-length HPR1 gene, an enzymatic activity-dead HPR1 gene (RCAS-DM-HPR1, double mutations at amino acid residues 225 & 343), and a C-terminus gene fragment (RCAS-8C, with a fusion of 8-kDa and the C-terminus of HPR1 including amino acid residues from 415-543). All inserts were tagged with a Myc epitope. This allowed us to titrate virus concentrations and monitor the expression levels in vivo. Western blot analysis with an anti-Myc tag antibody revealed that HPR1 was detected as an 18-kDa protein in DF-1 cells transfected with RCAS-8C vector, whereas the full-length HPR1 was detected as 50-kDa protein. Immunofluorescence staining revealed that RCAS-HPR1 virus-infected DF-1 cells had lower cell surface heparan sulfate levels, compared to RCAS-8C-infected DF-1 cells. These results confirmed that the C terminus of HPR1 did not have HPR1 enzymatic activity.

We next determined if the full-length HPR1 and enzymatically inactive HPR1 were functional in stimulating the PI-3 kinase pathway. KAT-18 cells (a thyroid tumor cell line), NIH 3T3 murine

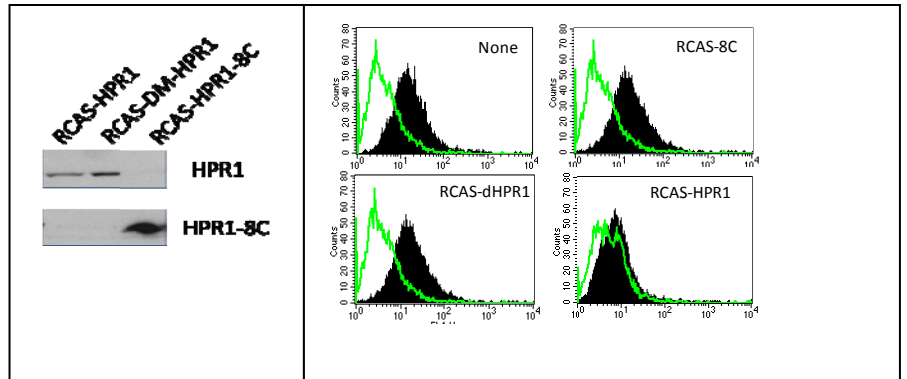


Fig. 1. HPR1 expression and cell surface HS levels. DF-1 cells were infected with RCAS-HPR1 (left), RCAS-DM-HPR1 (middle) or RCAS-HPR1-8C. After incubation for 48 hr, the cells were harvested and analyzed for HPR1 expression by Western blot with an anti-Myc epitope antibody or for cell surface HS levels by staining with an anti-HS IgM mAb followed by FACS analysis. Green line, isotype control; Black area. anti-HS IgM.

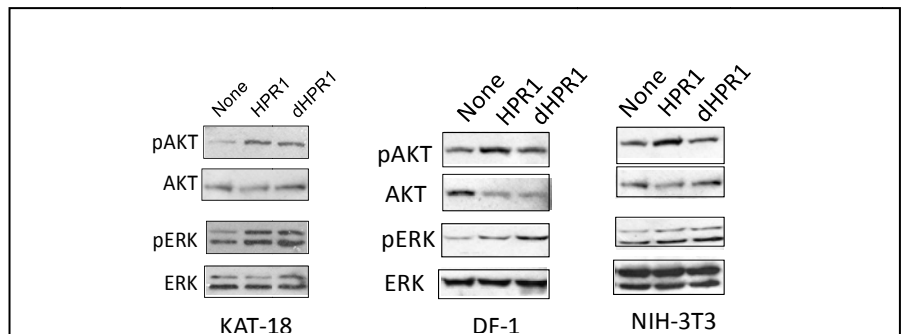
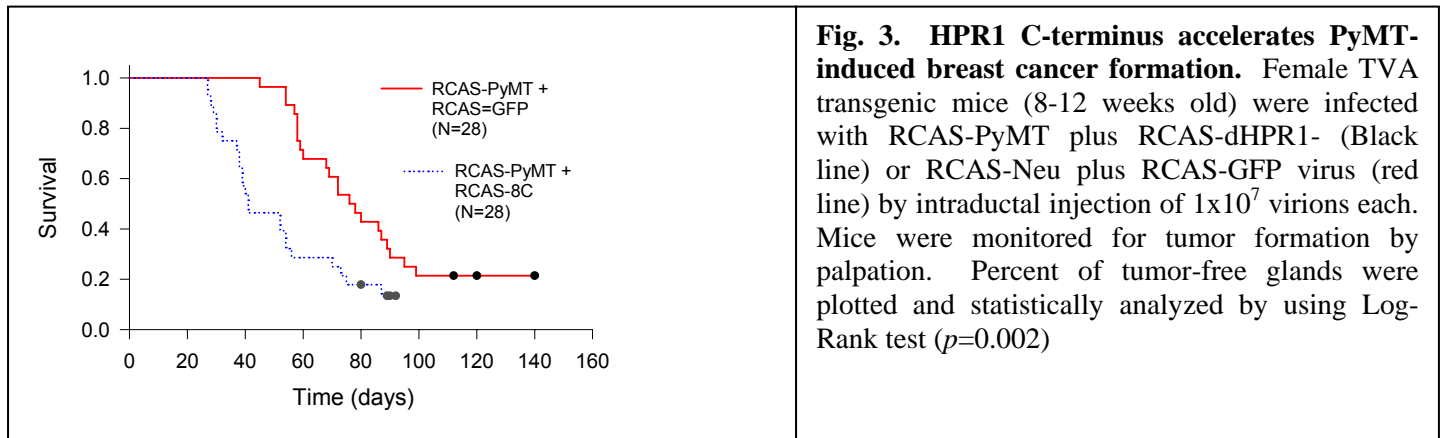


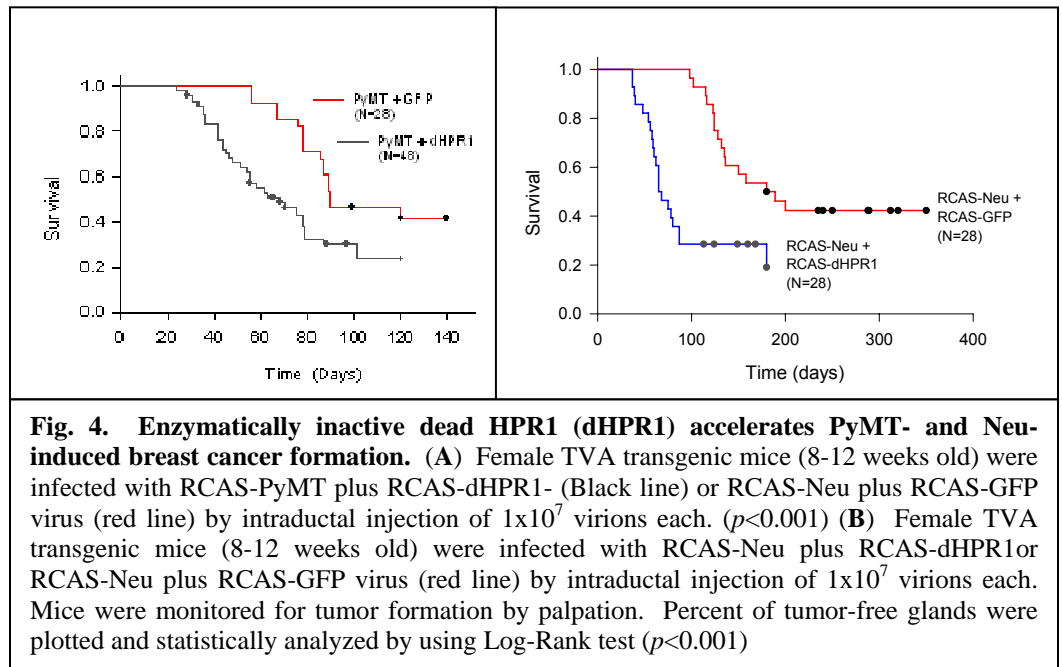
Fig. 2. The effect of HPR1 on AKT and ERK phosphorylation. KAT-18, DF-1, and NIH-3T3 cells were starved in the medium containing 0.5% FBS overnight. The cells were then left untreated or treated for 30 min with HPR1 or enzymatically dead HPR1 (dHPR1) purified via heparin-agarose beads from the conditioned media of DF1 cells infected with RCAS retroviral vector. AKT and ERK phosphorylation was detected by their specific antibodies. Antibodies against total proteins were used as control.

fibroblast cells, and DF-1 chicken fibroblast cells were pre-starved in serum-free medium overnight and then stimulated with purified HPR1 and dHPR1. As expected, both forms of HPR1 induced AKT phosphorylation in KAT-18 cells but were slightly less effective in inducing AKT phosphorylation in two fibroblast cells (Fig. 2). Interestingly, both forms of HPR1 stimulated ERK phosphorylation in KAT-18 cells. Of note, only p42 ERK was detected in chicken fibroblast cells with the anti-ERK antibodies.

The effect of HPR1 on tumor latency. We originally proposed to use TVA transgenic mice intercrossed with MMTV-PyMT, Neu, or Wnt transgenic mice. We have changed these experimental procedures by co-infecting TVA transgenic mice with RCAS vectors encoding the oncogene PyMT, Neu or Wnt plus the control RCAS vector or the vector encoding RCAS-8C. As shown in Fig. 3, mice infected with RCAS-PyMT plus RCAS-8C vectors developed breast cancer significantly faster than mice infected with RCAS-PyMT plus RCAS-GFP vector. The mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP were 86 ± 6 days versus 52 ± 4 days in TVA transgenic mice infected with RCAS-PyMT plus RCAS-8C. Statistical analysis revealed that the C terminus of HPR1 significantly accelerated breast formation induced by PyMT.



We next determined if an enzymatically inactive dead HPR1 was able to accelerate breast tumor initiation. As shown in Fig. 4, TVA transgenic mice infected with RCAS-PyMT or RCAS-Neu plus RCAS-dHPR1 vectors developed breast cancer much faster than mice infected with RCAS-PyMT or RCAS-Neu plus a control RCAS-vector, respectively. The mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-dHPR1 were 89 ± 6 versus 63 ± 5 days in TVA transgenic mice infected with RCAS-PyMT plus RCAS-GFP. The mean tumor latency in TVA transgenic mice infected with RCAS-Neu plus RCAS-dHPR1 were 95 ± 11 versus 228 ± 21 days in TVA transgenic mice infected with RCAS-Neu plus RCAS-GFP. These observations strongly suggested that that HPR1 enzymatic activity is disposable for its tumor promoting effect.



Finally, we determine the effect of full-length HPR1 in breast cancer initiation. TVA transgenic mice infected with RCAS-PyMT plus RCAS-HPR1 vectors developed breast cancer significantly slower than mice infected with RCAS-PyMT plus a RCAS-GFP vector. As shown in Fig. 5, the mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-HPR1 were 119 ± 8 days, whereas the mean tumor latency in TVA transgenic mice infected with RCAS-PyMT plus RCAS-GFP were 86 ± 6 . TVA transgenic mice co-infected with RCAS-PyMT and RCAS-HPR1 significantly prolonged breast cancer formation. Studies are ongoing to determine if HPR1 also accelerates Neu-induced breast formation.

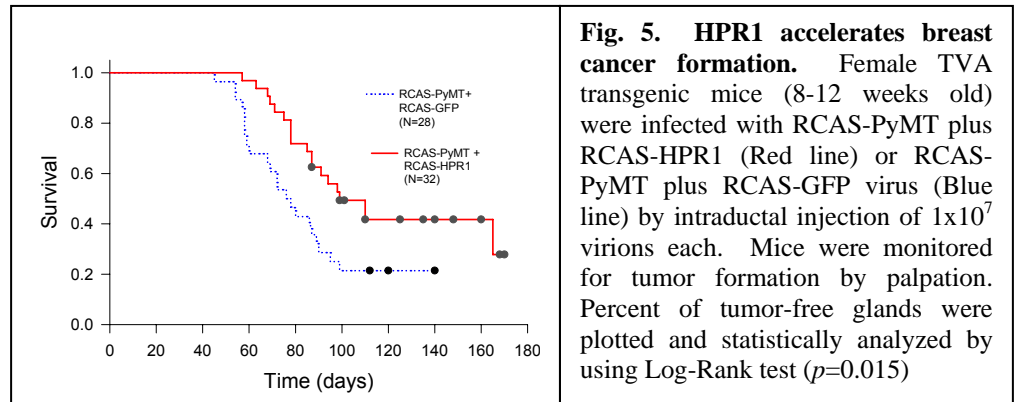


Fig. 5. HPR1 accelerates breast cancer formation. Female TVA transgenic mice (8-12 weeks old) were infected with RCAS-PyMT plus RCAS-HPR1 (Red line) or RCAS-PyMT plus RCAS-GFP virus (Blue line) by intraductal injection of 1×10^7 virions each. Mice were monitored for tumor formation by palpation. Percent of tumor-free glands were plotted and statistically analyzed by using Log-Rank test ($p=0.015$)

KEY RESEARCH ACCOMPLISHMENTS:

Task 2. To determine whether HPR1 enzymatic activity suppresses breast tumor initiation (Year 2)

1. Demonstrated that HPR1 C-terminus was able to promote breast cancer formation induced by PyMT oncogene
2. Demonstrated that the enzymatic activity of HPR1 had a negative effect on breast cancer formation induced by Neu and PyMT oncogene
3. Demonstrated that the enzymatically inactive HPR1 accelerated breast cancer formation induced by both RCAS-Neu and PyMT oncogenes.

REPORTABLE OUTCOMES

Manuscript: Domain-specific tumor-promoting activity of heparanase. Manuscript in preparation

CONCLUSION

We proposed to determine if HPR1 enzymatic activity can antagonize the tumor promoting effect of the C terminus of HPR1. Our studies using a syngeneic breast cancer model and somatic mouse model suggest that knockdown of HPR1 expression causes the delay of tumorigenesis and the inhibition of tumor growth, suggesting the overall role of HPR1 is to promote tumor growth and formation, and that the C terminus of HPR1 alone was able to promote tumor initiation and growth induced by Neu oncogene in a somatic breast cancer model. Studies in the past year demonstrated that the C-terminus of HPR1 accelerated breast cancer formation induced by PyMT. In contrast, the full-length HPR1 not only delayed breast cancer initiation. In contrast, enzymatically dead HPR1 accelerated breast cancer formation. These results are consistent with our earlier observations that sulodexide, an HPR1 inhibitor, accelerated breast cancer formation. Our results collectively suggest that inhibition of HPR1 activity not only does not suppress but rather accelerate breast cancer initiation, and that the strategy using a specifically inhibitor of HPR1 enzyme could have an adverse effect on cancer relapse.

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